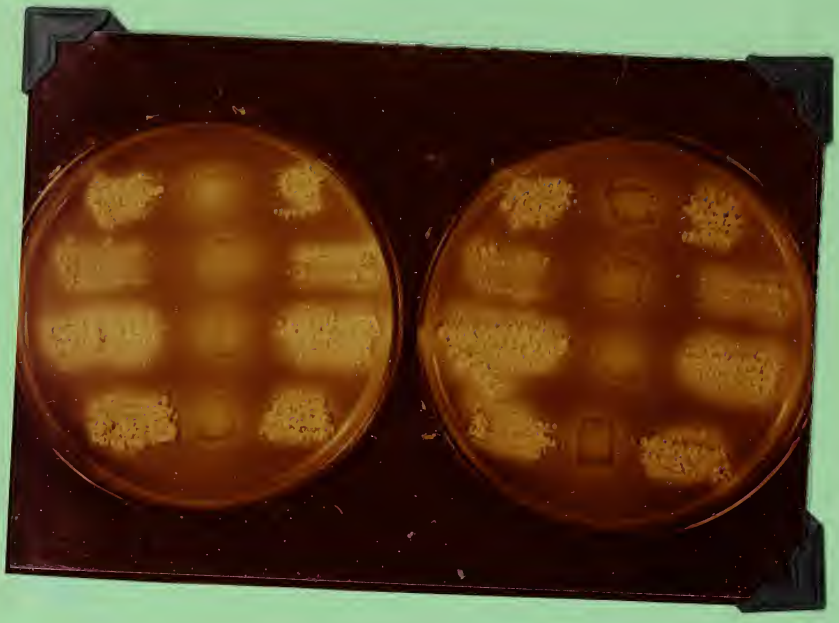
THE INHIBITION OF THE GROWTH OF MYCOBACTERIUM BY STAPHYLOCOCCUS

by
Gordon Edward Myers,
Bachelor of Science in Pharmacy



KODACHROME FRONTISPIECE

Staphylococcus versus *Mycobacterium*

(see text page 18)

THE INHIBITION OF THE GROWTH OF *MYCOBACTERIUM* BY *STAPHYLOCOCCUS*

by

Gordon Edward Myers,

Bachelor of Science in Pharmacy

A thesis submitted from the Department of Bacteriology and Hygiene in partial fulfilment of the requirements for the degree of Master of Science in Bacteriology at the University of Alberta.

ACKNOWLEDGEMENT

The writer wishes to express his appreciation to Dr. R.M. Shaw, Professor of Bacteriology and Director of the Provincial Laboratory and Dr. J.A. Romeyn, formerly Associate Professor of Bacteriology, University of Alberta, for their invaluable guidance during the course of this investigation and also to the Medical Section of the National Research Council, Ottawa, through whose interest and effort this study was partially financed.

Photographic plates accompaning this report were prepared at the Department of Extension, University of Alberta.

TABLE OF CONTENTS

TABLE OF CONTENTS

INTRODUCTION

THE INHIBITION OF THE GROWTH OF *MYCOBACTERIUM* BY *STAPHYLOCOCCUS*

INTRODUCTION

Knowledge of antagonistic interrelationships among micro-organisms dates back to the earliest attempts to isolate these organisms. Pasteur's discovery that microbes are responsible for certain human, animal and plant diseases, was followed closely by the observation that other organisms are able to combat and even destroy the disease-producing agents. The latter discovery opened up the field of microbial antagonisms and led to the study of the activities and potentialities of these antagonistic organisms. Little is actually known concerning the nature or the mode of production of the antibiotic substances they produce and even less is known of their mode of action.

Jennings and Sharp (1) in a recent review of the antibacterial activity of the *Staphylococcus* state that the first observation of this phenomenon was by Babes, who showed that a *Staphylococcus* inhibited the growth of *B. anthracis* and of another *Staphylococcus.* Over a period of sixty years some fifty papers have been published regarding this phenomenon. The majority of these reports have been concerned

with the action of Staphylococcus against the diphtheria bacillus.

In 1909 Schiøtz recorded the observation that a patient with a staphylococcus sore throat, nursed in a diphtheria ward because of a mistaken diagnosis, did not develop diphtheria, and that carriers became free of the diphtheria bacillus during a non-specific throat infection.

Dujardin-Baumetz described in 1932 (2) the inhibition of surface growth of saprophytic acid-fast bacilli and cold-blooded tubercle bacilli near growths of staphylococci. He did not study the effect on mammalian tubercle bacilli. He states that the diffusible inhibitory material is destroyed by 60°C. for one hour but not by 50°C. for two hours. Strains isolated from the nasal mucous membrane were active but apparently most strains isolated from other sources were not.

McPhedran, Lane and Crosson published in 1939 (3) an account describing marked anatomical and clinical improvement in cases of pulmonary tuberculosis treated with Staphylococcus vaccine. They state that such improvement only occurs as long as local reactivity to the Staphylococcus is present.

Arena in 1944 (4) demonstrated that a certain strain of Staphylococcus isolated from a "mucopurulent secretion from a non-tuberculous infection of the respiratory tract" would inhibit warm-blooded tubercle bacilli (type not stated) on coagulated egg medium. The two organisms were inoculated a few hours or days apart. He confirms the observation of Dujardin-Baumetz that not all strains show inhibitory activity.

Apart from the note by Jennings and Sharp, the most

recent study of the antibiotic activities of Staphylococcus in general appears to be that of Duliscouet published in late 1945 (5). Unfortunately this reference became available to us after our preliminary investigation was completed, consequently we have not been able to check Duliscouet's work in its entirity, nevertheless many of the characteristics described by Duliscouet correspond with our own results. The staphylococci used by Duliscouet were isolated from the throats of convalescent diphtheria patients and the diphtheria bacillus was the indicator of inhibitory capacity. Under certain circumstances the staphylococci are reported to increase the growth of diphtheria bacilli, however the majority of the strains tested inhibit the growth of diphtheria bacilli. Aureus, citreus and albus strains were shown to be active. The best organisms (classified on inhibitory potency) were rather larger and less Gram positive than usual, and the colonies were smaller and more delicate than usual. Mixed culture techniques are stated to have changed inactive to active strains.

Simultaneous inoculation of active staphylococci and diphtheria bacilli into the same site in guinea pigs protected them from death due to the latter: but if the injections were made into different sites there was no protection, and if diphtheria toxin was substituted for diphtheria bacilli there was no protection. Filtered cultures of staphylococci in liquid media had very little inhibitory activity. The active principle was best recovered from growths on solid media: when nutrient agar on which the organisms grew was dissolved in

water it was found to inhibit growth of diphtheria bacilli on solid and in liquid media. In vivo results were essentially the same. A number of characteristics of the active principle are mentioned, as follows: relative thermolability (destroyed in a few minutes at 80°C.), active in high dilutions, precipitable by alcohol, soluble in water and glycerine, absorbed readily by albuminoid materials, optimum pH for inhibition 7.5-8.5, and increased activity in the presence of calcium ions. The substance is thought to be an enzyme.

Jennings and Sharp (1) dealt with the effect of two hundred and five Staphylococcus strains on Staphylococcus aureus, Corynebacterium xerosis, Corynebacterium diphtheriae and Escherichia coli.

Surface plate tests were performed and twenty-six actively inhibiting Staphylococcus strains were found. C. xerosis was inhibited by all twenty-six active strains, Staphylococcus aureus and C. diphtheriae by most of the active strains and E. coli by none. None of pigment production, lactose fermentation, mannite fermentation or coagulase production were related to inhibitory potency.

Our interest in the action of Staphylococcus on Mycobacterium tuberculosis was aroused by certain clinical and pathological observations in a case of pulmonary tuberculosis which will be described below. The patient O. B. was a twenty-nine year old, male, white soldier. During 1944 while in England he developed periodic attacks of "bronchitis". So far as we know, X-ray and sputum studies in England were negative for tuberculosis. He became seriously ill in April, 1945 and for

weeks ran an almost continuous temperature of well over 100°F., often 105 and 106°F. He had copious sputum, but no tubercle bacilli could be demonstrated in smear and a guinea pig inoculation with the sputum was negative for tuberculosis. X-ray was inconclusive as to the presence of parenchymal tuberculosis. The sputum contained large numbers of Monilia (said to be non-pathogenic by a mycologist) and Staphylococcus. He developed a pleural effusion from which tubercle bacilli of human type were isolated.

The patient died in June, 1945. At autopsy, he had massive pleural tuberculosis, the pleurae being thickened as much as half an inch in places. There was remarkably little parenchymal involvement, amounting to slight local extensions just beneath the pleura in a few places.

It was felt that a man in such a debilitated condition over such a prolonged period, and with tuberculosis, should have shown more parenchymal involvement. For this reason tests were made of Monilia, Staphylococcus aureus, Streptococcus viridans and unidentified gram negative bacillus strains isolated from his sputum, to determine whether any of these organisms would inhibit the strain of tubercle bacilli isolated from the pleural fluid. The gram negative bacilli overgrew the plates and inhibition was not determined: of the others only the Staphylococcus aureus showed any activity. The investigation described in this report was based upon this initial observation.

PART ONE

TESTING OF STAPHYLOCOCCUS FOR INHIBITORY ACTIVITY

Introduction

Methods of testing antagonistic action have been described by Selman A. Waksman in his book on "Microbial Antagonisms and Antibiotic Substances" (6). Solid media have been used extensively for testing the action of antagonists. One of the methods most commonly used has been the simultaneous inoculation of antagonist and test organism. Waksman states that this method was introduced by Garré (7) in 1887. The method consists in streaking the antagonist and the test organism on the surface of solidified agar or gelatin medium. The streaks are alternate and may be parallel, radiating from a common centre, or intersecting at right angles. If the antagonistic agent produced does not diffuse for any considerable distance into the medium, the method is not satisfactory.

Experimental

In our initial work the following procedure was adopted. Two plates of Loewenstein medium (see Appendix 1) were used for each test of inhibitory potency of a *Staphylococcus* strain against a *Mycobacterium* strain. Twenty-five strains of *Staphylococcus*, collected from the Provincial

Laboratory routine and including strains from many sources, were tested against the following eleven strains of acid-fast bacilli.

Strain No.	Type	Source
1961H	Probably human	Sputum from Provincial Laboratory routine
2287H	Probably human	Sputum from Provincial Laboratory routine
221H	Human	A.T.C.C. 3236 (the H-37 strain)M
192H	Human MM	From pleural fluid of the case described previously
218B	Bovine	A.T.C.C. 9805 M
219B	Bovine	A.T.C.C. 9834 M
220B	Bovine	A.T.C.C. 599 M
222A	Avian	A.T.C.C. 7999 M
223A	Avian	A.T.C.C. 9077 M
224CB	Cold-blooded	A.T.C.C. 9819 M
225S	M. smegmatis	A.T.C.C. 101 M

Strains of acid-fast bacilli tested for inhibition by Staphylococcus

M Strains marked A.T.C.C. were obtained from the American Type Culture Collection, 2029 M Street, N. W., Washington 6, D. C.

MM This strain was typed by animal inoculation.

The method of testing was to inoculate the plates with the acid-fast organisms in such a manner that a heavy confluent growth would be produced on incubation. Immediately after inoculation of the acid-fast organisms an inoculum of the strain of Staphylococcus to be tested was streaked in a straight line down the centre of the plate on top of the acid-fast inoculum. The plates were incubated for three or four days at 37°C., then sealed with Scotch tape to prevent drying of the medium and incubation at 37°C. was continued for the duration of the test.

Plates prepared as described above were examined every other day for the first two weeks of incubation, every third or fourth day for the next two weeks, and once a week for the remainder of the period of incubation. The slower growing strains were held as long as fifteen weeks in certain instances before maximum growth was considered to have been reached.

Inhibition was quantitatively estimated by the distance in centimetres from the edge of the Staphylococcus growth (and at right angles to it) to the nearest acid-fast growth. Duplicate plates gave the same results within 0.2 centimetres. The ease of obtaining similar results in the duplicate plates was rather striking, considering the method of inoculation.

The results obtained in these tests are presented in Figures 1 and 2. These results were obtained from the observation of some 580 test plates, prepared and observed as described above.

FIGURE ONE

The results shown in this Figure represent the "inhibitory distance" at its maximum, during the period of incubation.

Staphylococcus strains are arranged roughly in order of increasing inhibitory power from right to left.

Mycobacterium strains are arranged roughly in order of increasing susceptibility from top to bottom.

Figure One

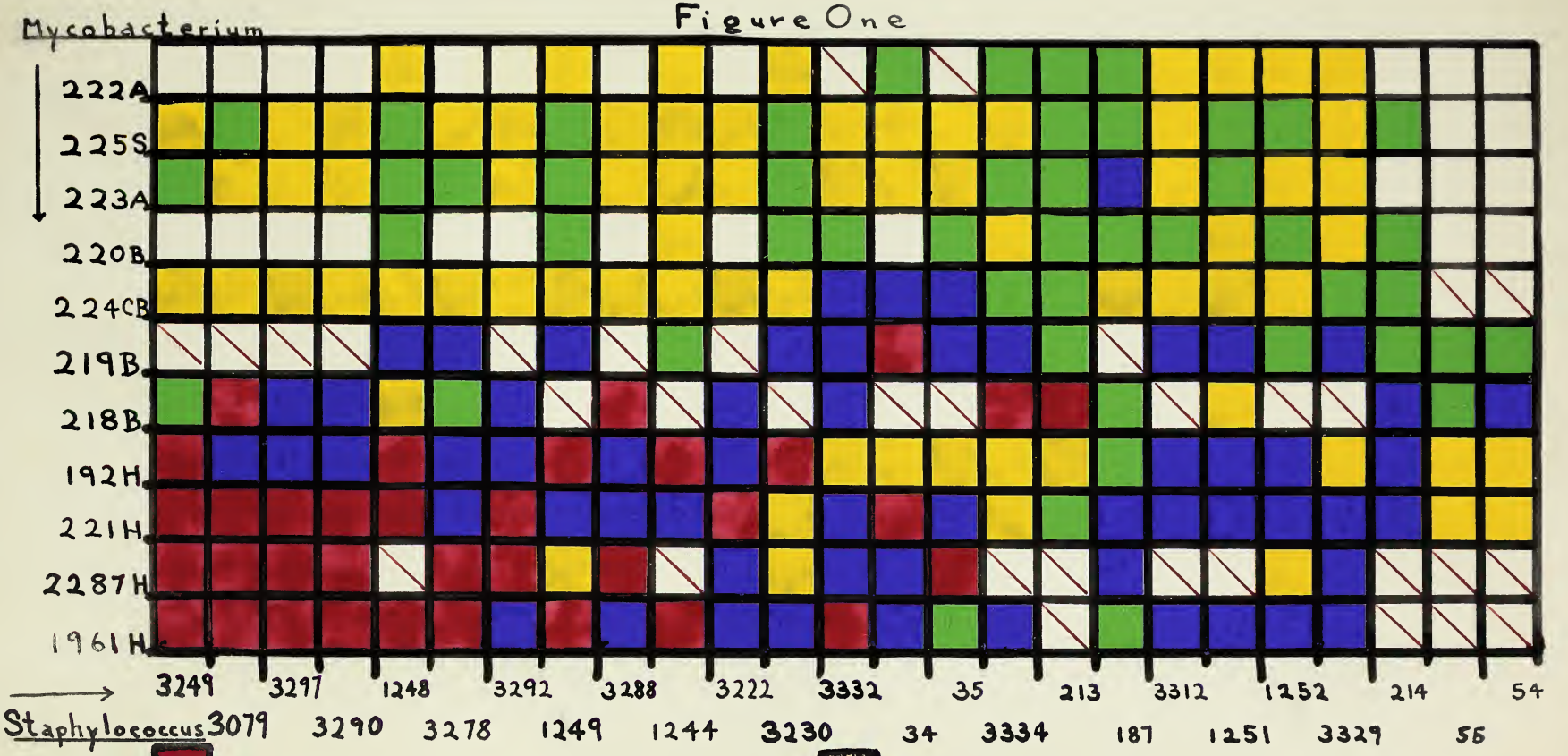

Mycobacterium
222A
225S
223A
220B
224CB
219B
218B
192H
221H
2287H
1961H
3249 3297 1248 3292 3288 3222 3332 35 213 3312 1252 214 54
Staphylococcus 3079 3290 3278 1249 1244 3230 34 3334 187 1251 3329 55
2.1-3.0 cms. inhibition
1.1-2.0 cms. "
0.1-1.0 cms. "
No inhibition
Growth over Staphylococcus
No data available

FIGURE TWO

The results shown in this Figure represent the "inhibitory distance" measured at the time of maximum growth.

Explanation:

The part of the acid-fast growth nearest the Staphylococcus growth had sometimes a decreased rate of growth so that the "inhibitory distance" decreased as the culture aged.

Staphylococcus strains are arranged roughly in order of increasing inhibitory power from right to left.

Mycobacterium strains are arranged roughly in order of increasing susceptibility from top to bottom.

Figure Two

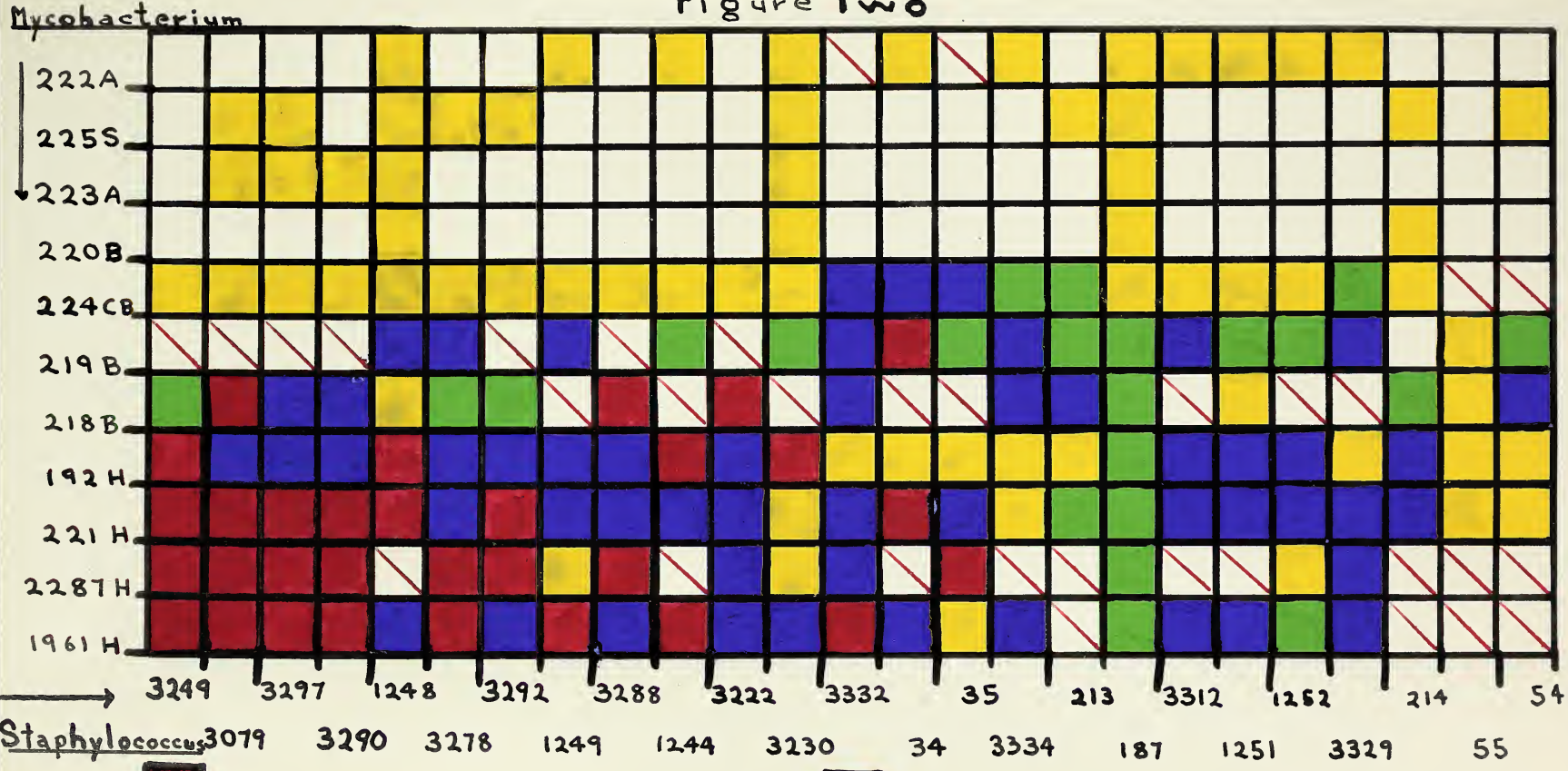

Mycobacterium
222A
225S
223A
220B
224CB
219B
218B
192H
221H
2287H
1961H
3249
3297
1248
3292
3288
3222
3332
35
213
3312
1252
214
54
Staphylococcus
3079
3290
3278
1249
1244
3230
34
3334
187
1251
3329
55
2.1-3.0 cms. inhibition
1.1-2.0 cms. "
0.1-1.0 cms. "
No inhibition
Growth over Staphylococcus
No data available

CHART ONE

This chart gives the actual measurements of inhibition used in making up Figure One.

CHART ONE

A.F.B. / Staph.	1961H	2287H	221H	192H	218B	219B	224CB.	220B	223A	225S	222A
3249	3.0	3.0	2.5	2.8	1.0		0	00	0.1	0	00
3079	3.0	3.0	3.0	1.8	3.0		0	00	0	0.2	00
3297	2.5	2.8	2.5	2.0	1.7		0	00	0	0	00
3290	2.6	3.0	2.7	1.5	2.0		0	00	0	0	00
1248	2.2		2.5	2.5	0	1.8	0	0.1	0.2	0.2	0
3278	2.2	3.0	2.0	1.5	0.5	1.5	0	00	0.1	0	00
3292	2.0	2.5	2.5	1.5	1.5		0	00	0	0	00
1249	2.6	0	1.5	2.5		1.8	0	0.3	0.3	0.1	0
3288	1.8	2.5	2.0	1.5	3.0		0	00	0	0	00
1244	2.5		1.4	2.5		0.8	0	0	0	0	0
3222	1.5	1.5	2.5	1.5	2.0		0	00	0	0	00
3230	2.0	0	0	3.0		1.2	0	0.5	0.2	0.2	0
3332	2.8	2.0	2.0	0	1.5	1.8	2.0	0.1	0	0	
34	2.0	2.0	2.5	0		2.8	1.8	00	0	0	0.3
35	0.2	3.0	2.0	0		1.5	1.7	0.1	0	0	
3334	2.0		0	0	2.5	1.5	1.0	0.5	0.5	0	0.2
213			1.0	0	2.5	1.0	0.5	0.5	0.5	0.2	0.3
187	0.5	1.5	1.3	1.0	0.3		0	1.0	1.1	0.8	0.3
3312	2.0		2.0	2.0		1.8	0	0.2	0	0	0
1251	1.8		2.0	2.0	0	1.3	0	0	0.2	0.2	0
1252	1.5	0	1.8	2.0		1.0	0	0.3	0	0.1	0
3329	1.8	2.0	1.4	0		1.5	0.1	0	0	0	0
214			1.5	2.0	1.5	0.8	0.5	0.5	00	0.2	00
55			0	0	1.0	0.5		00	00	00	00
54			0	0	2.0	0.4		00	00	0	00

All strains of acid fast bacilli arranged in order of speed of growth, slowest to the left.

All strains of Staphylococcus arranged in order of inhibition, greatest uppermost.

Symbols:

A blank---No reading possible for one or both of the following reasons,

(a) No acid fast growth visible
(b) Contaminated plate.

OO---------Acid fast growth completely covered Staphylococcus growth

O----------Acid fast growth right up to but not over Staphylococcus growth

Numerals:--Measurement of inhibition in centimetres (see text-page nine)

CHART TWO

This chart gives the actual measurements of inhibition used in making up Figure Two.

CHART TWO

A.F.B.	1961H	2287H	221H	192H	218B	219B	224CB.	220B	223A	225S	222A
Staph. 3249	3.0	3.0	2.5	2.5	0.8		0	00	00	00	00
3079	3.0	3.0	3.0	1.8	3.0		0	00	0	0	00
3297	2.5	2.8	2.5	2.0	1.3		0	00	0	0	00
3290	2.6	3.0	2.5	1.3	2.0		0	00	0	00	00
1248	1.8		2.3	2.3	0	1.7	0	0	0	0	0
3278	2.2	3.0	1.8	1.5	0.4	1.5	0	00	00	0	00
3292	2.0	2.5	2.5	1.5	1.0		0	00	00	0	00
1249	2.2	0	1.5	2.0		1.7	0	00	00	00	0
3288	1.7	2.5	2.0	1.5	3.0		0	00	00	0	00
1244	2.5		1.3	2.2		0.3	0	00	00	00	0
3222	1.2	1.3	2.0	1.5	2.2		0	00	00	00	00
3230	1.7	0	0	2.5		0.8	0	0	0	0	0
3332	2.8	2.0	1.8	0	1.4	1.8	1.5	00	00	00	
34	1.8		2.4	0		2.8	1.5	00	00	00	0
35	0	3.0	1.8	0		1.0	0.4	00	00	00	
3334	1.8		0	0	1.7	1.5	0.7	00	00	00	0
213			0.4	0	2.0	0.2	0.4	00	00	0	00
187	0.5	0.8	0.7	0.8	0.3	0.8	0	0	0	0	0
3312	1.8		1.5	2.0		1.4	0	00	00	00	0
1251	1.5		2.0	2.0	0	0.8	0	00	00	00	0
1252	1.0	0	1.8	2.0		0.3	0	00	00	00	0
3329	1.3	1.4	0.4	0		1.4	0.4	00	00	00	0
214			1.5	2.0	1.0	00	0	0	00	0	00
55			0	0	0	0		00	00	00	00
54			0	0	1.3	0.4		00	00	0	00

All strains of acid fast bacilli arranged in order of speed of growth, slowest to the left.

All strains of Staphylococcus arranged in order of inhibition, greatest uppermost.

Symbols:

A blank---No reading possible for one or both of the following reasons,

(a) No acid fast growth visible
(b) Contaminated plate.

OO--------Acid fast growth completely covered Staphylococcus growth

O---------Acid fast growth right up to but not over Staphylococcus growth

Numerals:-- Measurement of inhibition in centimetres(see text-page nine)

Duplication of Results:

The remarkable duplication of results obtainable is illustrated in the Kodachrome Frontispiece. Two plates of Loewenstein medium are depicted. The plates were inoculated with four human strains of Mycobacterium tuberculosis in parallel transverse surface streaks, an area one centimetre square in the middle of each streak was immediately inoculated with a strain of Staphylococcus. The four human strains of Mycobacterium tuberculosis as well as the strain of Staphylococcus shown in these duplicate plates were among those tested in the investigation with which this report is concerned. This modification of the plate technique was developed with a view to using less medium for the tests. Formerly we had used one plate of Loewenstein medium for each strain of Mycobacterium tested. This latest technique made it possible to test four strains of Mycobacterium on each plate of medium. When we take into consideration the necessity of duplicate plates for each test we see that this technique reduces the number of plates required to test four strains of Mycobacterium from sixteen to two. The method of inoculating a small area in the middle of each streak of Mycobacterium with the strain of Staphylococcus being tested was employed in preference to inoculating the Staphylococcus strain in a continuous streak across the four streaks of Mycobacterium in order to avoid the dragging of organisms from one acid-fast inoculum to another. One previously tested strain of Staphylococcus was tested using this method and gave the same results as when

tested by the initial technique (one year previously). This modification was adopted as the routine method of testing for inhibitory activity.

Organisms other than Staphylococcus tested for Inhibitory Potency:

Three strains of haemolytic streptococci from throats, one diphtheroid bacillus, one unidentified gram positive bacillus and one unidentified strain of Actinomyces were tested. Two of the haemolytic streptococcus strains slowed the growth of one human strain of Mycobacterium tuberculosis and one of them prevented overgrowth by the human acid-fast bacilli but not by the other strains of acid-fast bacilli. Otherwise no inhibition was observed.

Various Types of Relationship between Staphylococcus strains and Mycobacterium strains:

Figures Three to Nine illustrate the various types of relationship between strains of Staphylococcus and Mycobacterium as observed, when these organisms were tested by the methods described.

FIGURE THREE

Staphylococcus, golden, haemolytic -- # 3278

M. avium -- A.T.C.C. # 9077

Inoculated: August 9, 1946

Photographed: September 5, 1946

Medium: Loewenstein

FIGURE FOUR

Staphylococcus, golden, haemolytic -- # 3312

M. tuberculosis (var. hominis) -- # 192 -- Colonel Belcher Hospital

Inoculated: August 9, 1946

Photographed: September 5, 1946

Medium: Loewenstein

FIGURE 3

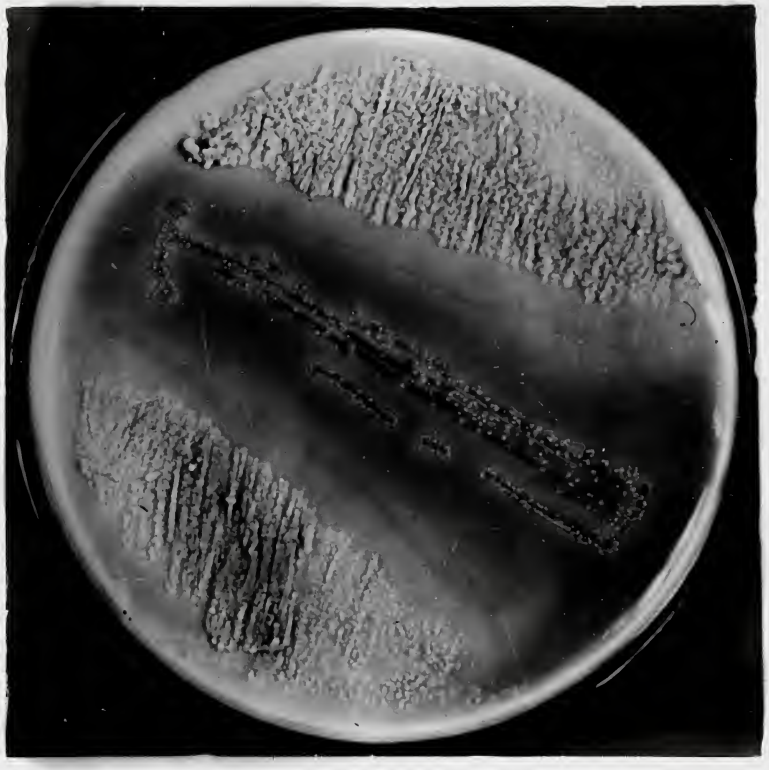


FIGURE 4

See text for explanation

FIGURE FIVE

Staphylococcus, golden, haemolytic -- # 3312

M. tuberculosis (probably human) -- # 1961

Inoculated: August 9, 1946

Photographed: September 5, 1946

Medium: Loewenstein

FIGURE SIX

Staphylococcus, golden, haemolytic -- # 1244

M. tuberculosis (probably human) -- # 1961

Inoculated: August 9, 1946

Photographed: September 5, 1946

Medium: Loewenstein

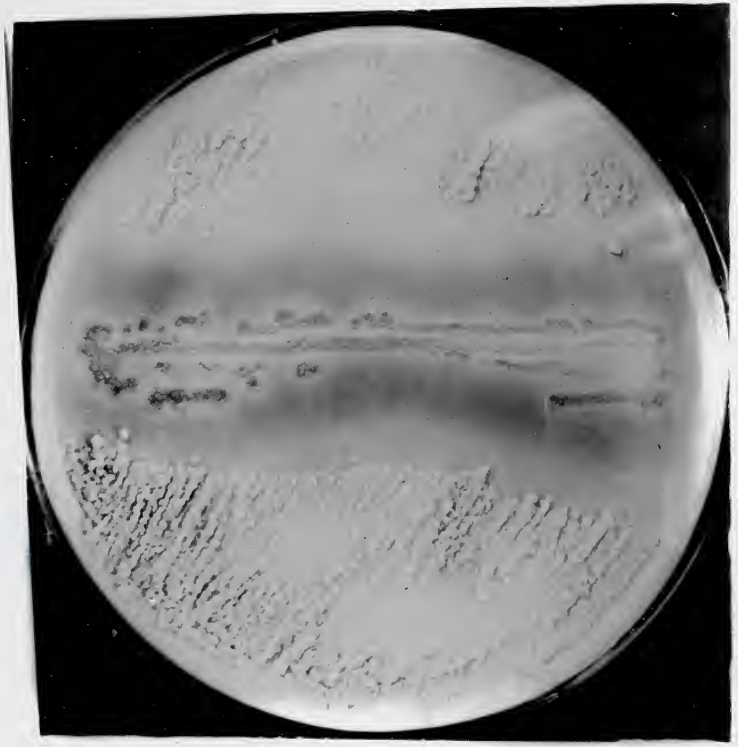


FIGURE 5

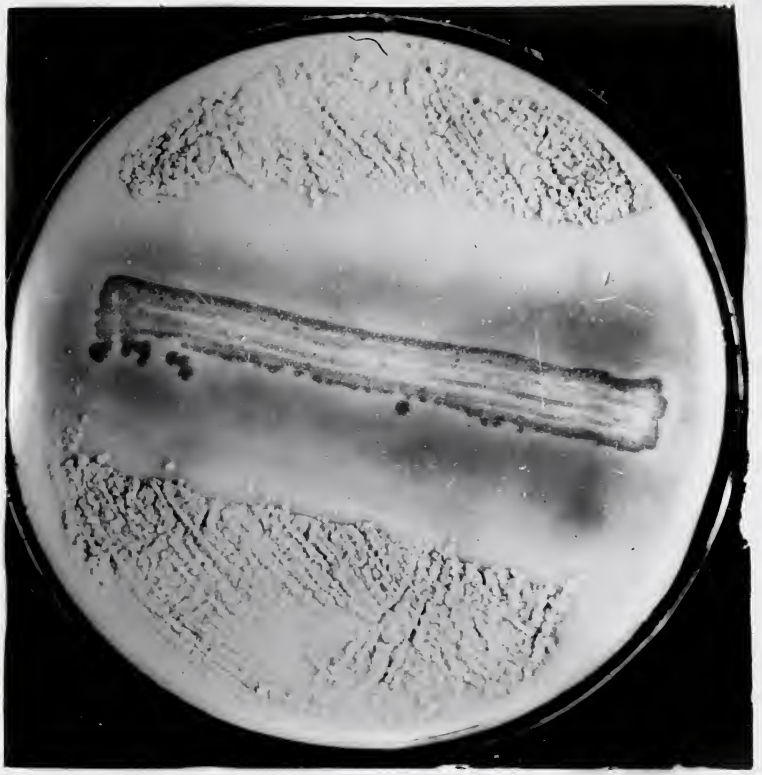


FIGURE 6

See text for explanation

FIGURE SEVEN

Staphylococcus, golden -- # 185

Top to bottom:

M. tuberculosis (probably human) -- # 1961

M. tuberculosis (probably human) -- # 2287

M. tuberculosis (var. hominis) -- A.T.C.C. -- # 8236

M. tuberculosis (var. hominis) -- Colonel Belcher Hospital

Inoculated: June 12, 1947

Photographed: August 6, 1947

Medium: Loewenstein

FIGURE EIGHT

Staphylococcus, golden -- # 185

Top to bottom:

M. tuberculosis (var bovis) A.T.C.C. -- # 9834

M. tuberculosis (cold-blooded) A.T.C.C. -- # 9819

M. tuberculosis (var bovis) A.T.C.C. -- # 599

Inoculated: June 12, 1947

Photographed: August 6, 1947

Medium: Loewenstein

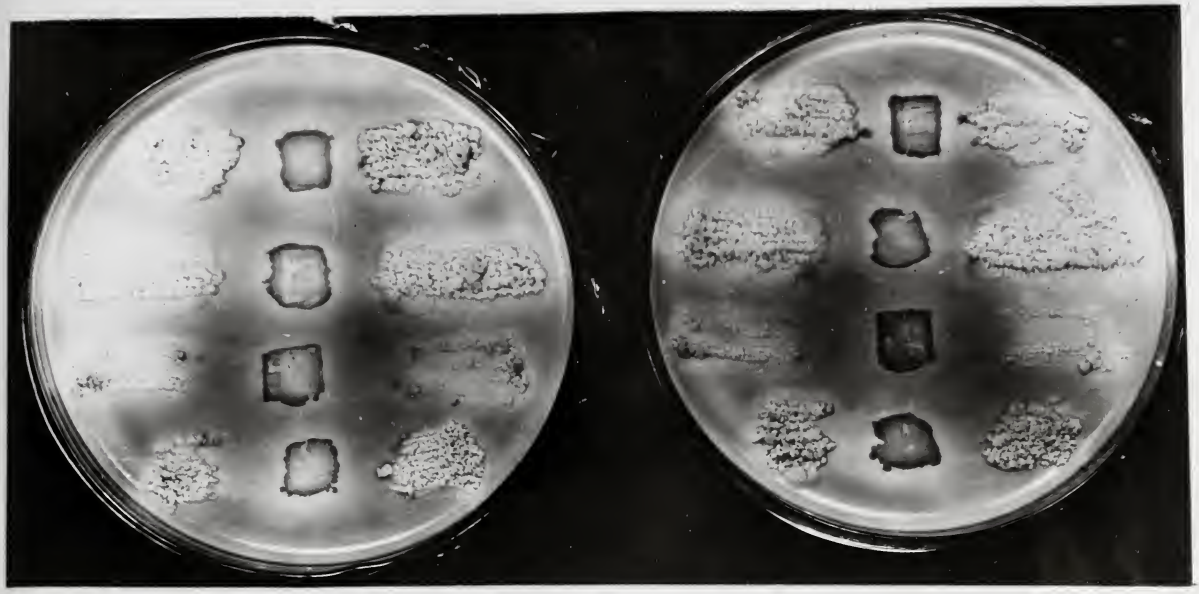


FIGURE 7

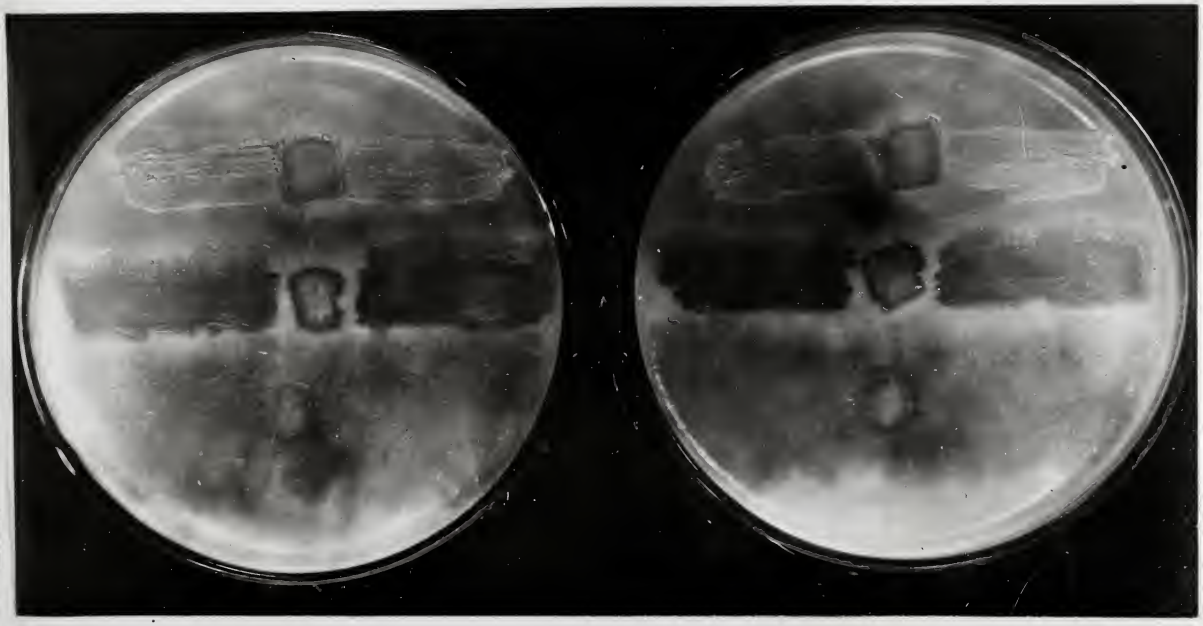


FIGURE 8

FIGURE NINE

Staphylococcus, golden, haemolytic -- # 185

Top to bottom:

M. tuberculosis (avian) A.T.C.C. -- # 9077

M. smegmatis A.T.C.C. -- # 101

M. tuberculosis (avian) A.T.C.C. -- # 7999

Inoculated: June 12, 1947

Photographed: August 6, 1947

Medium: Loewenstein

Certain Phenomena Observed with regard to Inhibition:

Test plates prepared for the purpose of estimating the inhibitory potency of various strains of Staphylococcus towards the growth of various strains of Mycobacterium often gave rise to certain unexplained peculiarities of growth which we have attempted to illustrate in Chart Three. The drawings shown in Chart Three were made directly from plates observed and irregularities in growth shown are as they appeared on such plates.

CHART THREE

Explanation:

Plate 1:

This plate shows the normal appearance of confluent surface growth of a strain of Mycobacterium (orange pigment) on Loewenstein medium, (blue-green background).

Plate 2:

This plate represents the appearance of the confluent surface growth of a strain of Mycobacterium (orange pigment) on Loewenstein medium (blue-green background) with a diametric surface streak of Staphylococcus growth (golden pigment). The area of inhibition is clearly seen between Mycobacterium and Staphylococcus growth. This was a typical plate.

Plates 3, 4 and 5:

These plates represent certain unexplained peculiarities of the growth of Mycobacterium observed.

The green coloration of the Mycobacterium growth demonstrated on Plates 4 and 5 was observed only on plates which had been subjected to prolonged incubation at 37°C. It is our opinion that the Mycobacterium growth has taken up the Malachite Green which is contained in the medium (see Appendix 1).

Chart Three

1

3

2

4

5

Growth Characteristics of Mycobacterium strains tested:

The character of the strains of Mycobacterium grown on Loewenstein medium followed closely the text-book descriptions of these strains.

Mycobacterium strains of the human type grew with the usual granular (bread crumb-like) colonial morphology on this medium. The strain 2287H produced a slightly more slime-like growth. The avian, bovine and cold-blooded strains gave rise to moist viscous confluent growth on Loewenstein medium. M. smegmatis produced dry granular confluent growth on the medium.

With regard to the pigmentation of the strains of Mycobacterium tested, the human types in general produced an orange-pink pigment, the avian and bovine strains a pinkish cream pigment, the cold-blooded strain a vivid yellow pigment and M. smegmatis produced a salmon pink pigment.

Growth Rates of Various Mycobacterium strains tested:

Mycobacterium strains used in this investigation were maintained on Loewenstein slants throughout the period of testing. Strains were transferred every four to six weeks, incubated at 37°C. until profuse growth appeared and then held at room temperature (20-25°C.). A record was kept during the tests and transfers to determine the average number of days of incubation at 37°C. required for a given strain of Mycobacterium to produce macroscopic colonies at 37°C. and the results of twenty-four transfers are given in Chart Four.

CHART FOUR

Explanation:

Mycobacterium strains are arranged in the column at the extreme left.

Transfer numbers are listed across the top of the chart.

Opposite each Mycobacterium strain will be found a number, which represents the number of days incubation at 37°C. required to give rise to macroscopic colonies for that transfer.

The column (A) at the extreme right gives the average number of days incubation at 37°C. required to give rise to macroscopic colonies for a given strain of Mycobacterium for the twenty-four transfers.

Example:

Mycobacterium strain 1961H required 11 days incubation at 37°C. to produce macroscopic colonies on the eighth transfer.

Mycobacterium strain 1961H required an average of 10 days incubation at 37°C. to produce macroscopic colonies, based on 24 transfers at four to six week intervals.

CHART FOUR

Strain:	Transfer Number: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	A
1961H	12	10	12	12	7	10	12	11	12	11	10	10	11	7	8	7	7	9	10	12	8	8	4	4	10
2287H	12	18	15	23	12	12	32	12	12	32	11	15	30	8	12	8	6	28	20	32	11	22	22	12	18
221H	12	23	12	12	15	12	12	11	12	11	12	11	15	15	30	15	11	15	8	8	6	8	15	11	14
192H	15	23	18	12	9	30	10	11	10	9	12	11	11	11	11	11	12	8	9	9	11	11	20	8	13
218B	10	12	8	12	22	10	10	12	10	15	15	14	8	15	23	18	28	16	10	14	7	8	12	24	14
219B	2	10	2	2	2	5	5	5	5	4	2	2	2	2	5	4	7	8	7	7	5	5	2	4	5
224CB	23	15	15	15	22	23	23	17	23	22	15	22	11	11	15	17	17	7	25	18	19	15	17	22	18
220B	2	2	2	2	1	2	2	1	2	1	2	1	1	1	1	1	2	2	1	1	1	1	2	2	2
223A	2	2	2	2	1	2	2	1	2	1	2	1	2	2	2	2	2	2	1	1	1	2	2	2	2
225S	2	2	2	2	1	2	2	1	2	1	2	1	2	2	2	2	2	2	1	1	1	1	2	2	2
222A	2	2	2	2	1	1	1	2	1	1	2	2	1	1	2	2	2	1	2	2	2	2	2	2	2

Explanation: The figure below the number of the Transfer, represents the number of days at 37°C. incubation required to give rise to macroscopic colonies, for the respective strain of Mycobacterium.

From the results shown in Chart Four we can arrange roughly the various strains of Mycobacterium in the order of rate of growth at 37°C. as follows:

Strain:	Average:
224CB	18 days at 37°C. Ⅿ
2287H	18 days at 37°C.
218B	14 days at 37°C.
221H	14 days at 37°C.
192H	13 days at 37°C.
1961H	10 days at 37°C.
219B	5 days at 37°C.
220B	2 days at 37°C.
223A	2 days at 37°C.
225S	2 days at 37°C.
222A	2 days at 37°C.

Ⅿ 224 CB the cold-blooded strain of Mycobacterium grows well at room temperature (20-25°C.), giving rise to macroscopic colonies within three to four days.

Summary

The relations between different forms of life vary with the environmental conditions of the moment. Our tests were done under only one set of environmental conditions and these were not entirely optimal for some, if not all, of the strains of bacteria used. The temperature of incubation was always 37°C., although the optimal temperatures for bacteria vary widely: the medium contained glycerine and Malachite Green, both of which had different effects, presumably, on the Staphylococcus and Mycobacterium strains used. These remarks merely emphasize the relativity of any observations made in this, or any, antibiotic work.

There are marked differences in the susceptibility to inhibition among the strains of acid-fast bacilli tested by this method. It would be a mistake to make too many generalizations on the basis of a study of so few strains. In general, however, the figures show that the human strains studied are more susceptible than the bovine strains, and the bovine more susceptible than the remainder of the strains tested.

In general, the fast-growing strains of Mycobacterium are not inhibited as much as the slow-growing strains, and often are not inhibited at all. It is not quite true, however, that if a strain is slow-growing, it will be inhibited: for instance, strain 224CB (growing at 37°C. well above its optimum 20-23°C., for it is a reptilian strain) is one of the slowest growing strains with which we worked, and yet it is very poorly inhibited.

A discussion of the various morphological and biochemical reactions of the strains of Staphylococcus concerned in this investigation will be given in PART TWO of this report.

PART TWO

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF STAPHYLOCOCCUS

Introduction

"In the earlier work on antagonisms, among micro-organisms, two main procedures were followed; the indiscriminate testing of pure cultures of bacteria and fungi, commonly taken from culture collections, for antagonistic activity against one another or against certain specific test organisms; and isolation of occasional antagonistic organisms from old plate cultures, as contaminants from the air, or from mixed infections. Neither of these methods is suitable where a systematic study of antagonism is desired. Recently a more systematic study of the distribution of antagonistic organisms in nature has been attempted. The work of Fleming (8) and other British workers (9, 10, 11), on the antibacterial properties of the molds belonging to the Penicillium notatum group served as a stimulus to the numerous studies that followed." The above quotation from Waksman (6) briefly describes the development of the study of microbial antagonism. Gradually the idea developed that in the case of bacterial antagonists one is dealing with specific strains rather than with distinct species. Stokes and Woodward (12) pointed out that the biochemical characteristics of spore-forming soil bacteria differed for strains possessing antagonistic properties and strains which did not possess these properties.

Pigment production has been related to antagonistic potency in the case of Ps. aeruginosa by Schoental (13). In any systematic study of the antagonistic properties of a given bacterial strain it therefore appears logical that a concurrent study of the morphological and biochemical characteristics of the strains of that species might demonstrate some correlation with the antagonistic properties and thus aid in the isolation of highly antagonistic strains.

Experimental

Our investigation of twenty-five strains of Staphylococcus dealt with the following characteristics: microscopic morphology and staining reaction, colonial morphology, pigmentation, haemolytic activity, lactose fermentation, mannite fermentation, coagulase production and a preliminary investigation of lipolytic activity. Before discussing the above mentioned characteristics perhaps we should note the source of the various strains. These data are given in Chart Five.

CHART FIVE

Source of *Staphylococcus*

CHART FIVE

Staphylococcus Strain No.	Source
3249	Isolated from wound (buttock)
3079	Isolated from food (poisoning ?)
3297	Unknown
3290	Isolated from eye
1248	Unknown
3278	Isolated from abscess
3292	Isolated from boil (face)
1249	Unknown
3288	Isolated from wound infection (thumb)
1244	Isolated from throat
3222	Unknown
3230	Isolated from infection (outer ear)
3332	Isolated from infection (hand)
34	Stock Culture (Provincial Laboratory)
35	Stock Culture (Provincial Laboratory)
3334	Isolated from infection (ear)
213	Stock Culture (Provincial Laboratory)
187	Isolated from sputum of patient O. B.
3312	Isolated from wound infection
1251	Unknown
1252	Unknown
3329	Isolated from abscess (knee)
214	Stock Culture (Provincial Laboratory)
55	Stock Culture (Provincial Laboratory)
54	Stock Culture (Provincial Laboratory)

Microscopic Morphology and Staining Reaction:

All strains of Staphylococcus examined in this investigation were of typical microscopic morphology being grape-like clusters of cocci, individual organisms approximately one micron in diameter. All strains examined stained Gram-positive. No significant variation in size, shape or staining characteristic was observed.

Colonial Morphology:

Growing on Nutrient agar medium and upon Blood agar medium all the strains of Staphylococcus tested produced typical circular, smooth colonies with entire edges. Isolated colonies of the various strains showed no significant variations in size, shape or consistency when growing on these media. Incubation temperature was 37°C. and time of observation for colonial characteristics was twenty-four hours in all cases.

Pigmentation:

Growing on Blood agar medium at 37°C. the pigment produced by the various strains of Staphylococcus is shown in Chart Six. Certain strains gave doubtful pigmentation at the end of twenty-four hours' incubation at this temperature and subsequent incubation and observation for forty-eight hours, seventy-two hours and ninety-six hours was carried out and recorded.

CHART SIX

Pigmentation of Staphylococcus

CHART SIX

Incubation Temperature 37°C.

Staphylococcus Strain No.	24 hours	48 hours	72 hours	96 hours
3249	Golden	Golden	Golden	Golden
3079	Golden	Golden	Golden	Golden
3297	Golden	Golden	Golden	Golden
3290	Golden	Golden	Golden	Golden
1248	Golden	Golden	Golden	Golden
3278	Golden	Golden	Golden	Golden
3292	White	White	White	White
1249	Golden	Golden	Golden	Golden
3288	Golden	Golden	Golden	Golden
1244	Golden	Golden	Golden	Golden
3222	Golden	Golden	Golden	Golden
3230	Golden	Golden	Golden	Golden
3332	Golden	Golden	Golden	Golden
34	White	White	White	White
35	White	White	White	White
3334	White	White	White	White
213	Golden?	Golden	Golden	Golden
187	Golden?	Golden	Golden	Golden
3312	Golden	Golden	Golden	Golden
1251	Golden	Golden	Golden	Golden
1252	Golden	Golden	Golden	Golden
3329	Golden	Golden	Golden	Golden
214	Golden?	White	White	White
55	Golden	Golden	Golden	Golden
54	White	White	White	White

Haemolytic Activity of Staphylococcus:

The various strains of Staphylococcus were tested for haemolytic activity on Blood agar medium, (Nutrient Agar containing 5% aseptically drawn, defibrinated sheep's blood) incubated at 37°C. Certain strains demonstrated delayed haemolytic action so that it was necessary to observe these plates at prolonged incubation periods. The results of these observations are outlined in Chart Seven.

CHART SEVEN

Haemolytic activity of Staphylococcus

CHART SEVEN

Incubation Temperature 37°C.

Staphylococcus Strain No.	24 hours	48 hours	72 hours	96 hours
3249	Haem.	Haem.Ⅱ	Haem.	Haem.
3079	Haem.	Haem.	Haem.	Haem.
3297	Haem.	Haem.	Haem.	Haem.
3290	Haem.	Haem.	Haem.	Haem.
1248	Neg.	Neg.	Sl.Haem.	Sl.Haem.
3278	Haem.	Haem.	Haem.	Haem.
3292	Haem.	Haem.	Haem.	Haem.
1249 No Test				
3288	Haem.	Haem.	Haem.	Haem.
1244	Haem.	Haem.	Haem.	Haem.
3222	Haem.	Haem.	Haem.	Haem.
3230	Haem.	Haem.	Haem.	Haem.
3332	Haem.	Haem.	Haem.	Haem.
34 No Test				
35	Sl.Haem.	Haem.Ⅱ	Haem.Ⅱ	Haem.
3334 No Test				
213	Haem.	Haem.	Haem.	Haem.
187	Neg.	Sl.Haem.	Haem.	Haem.
3312	Haem.	Haem.	Haem.	Haem.
1251	Haem.	Haem.	Haem.	Haem.
1252	Haem.	Haem.	Haem.	Haem.
3329	Haem.	Haem.	Haem.	Haem.
214	Haem.	Haem.	Haem.	Haem.
55	Haem.	Haem.	Haem.	Haem.
54 No Test				

Ⅱ Two zones of haemolysis observed.

Lactose Fermentation:

Strains of Staphylococcus under investigation were tested for lactose fermenting properties in beef-extract broth containing 1% lactose and 1% Andrade's indicator. Cultures were incubated at 37°C. for 96 hours and observed every 24 hours during that period for evidence of fermentation. All twenty-five strains of Staphylococcus tested fermented lactose, within 24 hours' incubation at 37°C., with the production of acid and without producing gas.

Mannite Fermentation:

Strains of Staphylococcus under investigation were tested for mannite fermentation in beef-extract broth containing 1% mannite and 1% Andrade's indicator. Cultures were incubated at 37°C. for 96 hours and observed every 24 hours during that period for evidence of fermentation. The results obtained for the twenty-five strains of Staphylococcus tested are presented in Chart Eight.

CHART EIGHT

Mannite fermentation by Staphylococcus

CHART EIGHT

Incubation Temperature 37°C.

Staphylococcus Strain No.	24 hours	48 hours	72 hours	96 hours
3249	Neg.	Acid	Acid	Acid
3079	Sl.Pos.	Acid	Acid	Acid
3297	Sl.Pos.	Acid	Acid	Acid
3290	Neg.	Acid	Acid	Acid
1248	Acid	Acid	Acid	Acid
3278	Acid	Acid	Acid	Acid
3292	Neg.	Acid	Acid	Acid
1249 No Test				
3288	Neg.	Acid	Acid	Acid
1244	Acid	Acid	Acid	Acid
3222	Acid	Acid	Acid	Acid
3230	Acid	Acid	Acid	Acid
3332	Acid	Acid	Acid	Acid
34 No Test				
35	Sl.Pos.	Acid	Acid	Acid
3334 No Test				
213	Acid	Acid	Acid	Acid
187	Neg.	Acid	Acid	Acid
3312	Acid	Acid	Acid	Acid
1251	Neg.	Acid	Acid	Acid
1252	Neg.	Acid	Acid	Acid
3329	Sl.Pos.	Acid	Acid	Acid
214	Acid	Acid	Acid	Acid
55	Acid	Acid	Acid	Acid
54 No Test				

Sl. Pos. -- Slight evidence of acid production.

Coagulase Production:

In their report on "The Differentiation of Pathogenic Staphylococci from Non-pathogenic Types", Chapman, Berens, Nilson and Curcio (14) state that with a few exceptions, any coagulating (i.e., Coagulase Positive) strain is pathogenic. These results are in accordance with the findings of previous workers. Different results have been obtained using this test in different laboratories and according to these workers, errors may be traced to at least three sources: the use of plasma from unsuitable animal species; the time of observing the results; and failure to recognize a minute coagulum. Our method of testing for coagulase production was that recommended by the above mentioned investigators. A loopful of the culture was obtained from solid medium and mixed with 0.5-1.0 c.c. of oxalated or citrated human plasma in 0.85% sodium chloride solution (equal parts of human plasma and normal saline solution). After shaking thoroughly, the mixture was allowed to stand at 37°C., and examined in 4 hours. If no coagulum could be detected the tubes were re-examined the following morning. The presence of a gelatinous globule in the tube was tested for by tilting the tube to the horizontal position and then back to the vertical position. The presence of any degree of clotting at either time of observation was considered as a positive test. The results obtained, for the twenty-five strains of Staphylococcus tested, are given in Chart Nine.

CHART NINE

Coagulase tests for Staphylococcus

CHART NINE

Incubation Temperature 37°C.

Staphylococcus Strain No.	Coagulase	
	Observation #1	Observation #2
3249	Positive	Positive
3079	Positive	Positive
3297	Positive	Positive
3290	Positive	Positive
1248	Positive	Positive
3278	Positive	Positive
3292	Positive	Positive
1249 No Test		
3288	Positive	Positive
1244	Positive	Positive
3222	Positive	Positive
3230	Positive	Positive
3332	Doubtful	Doubtful
34 No Test		
35	Negative	Negative
3334 No Test		
213	Positive	Positive
187	Positive	Positive
3312	Positive	Positive
1251	Positive	Positive
1252	Positive	Positive
3329	Positive	Positive
214	Positive	Positive
55	Negative	Negative
54	Negative	Negative

Preliminary Investigation of Lipolytic Activity of Staphylococcus

In 1901 Eijkman (15) devised a differential plating medium for lipase-producing bacteria. Since that time numerous references have appeared in the literature describing methods for the differentiation of lipolytic from non-lipolytic bacteria. Turner (16) reporting on the various techniques developed for this purpose states that Nile-blue sulphate medium containing an emulsion of tributyrin gave remarkable sharpness of differentiation and high degree of sensitivity, but inhibited the growth of certain organisms (namely certain strains of Clostridium welchii and Staphylococcus aureus). Collins and Hammer (17) consider the addition of Nile-blue sulphate to a nutrient agar medium containing dispersed fat provides a convenient method for the detection of bacteria that hydrolyze fat. The medium employed by Collins and Hammer was a beef-infusion agar adjusted to pH 6.8 to 7.0. To each 100 ml. volume of this base was added 10 ml. of a 0.1% aqueous solution of Nile-blue sulphate. The agar was then placed in tubes and sterilized. Fat to be used was prepared by filtration through a hot water funnel and addition to a 0.5% agar solution in the proportion of 10 ml. of fat to 90 ml. of the agar solution. This mixture was sterilized at 15 pounds for twenty-five minutes, cooled until it was nearly solidified and then vigorously shaken to ensure an emulsion of fat. Just before use the fat emulsion was heated to a temperature that would give a soft jelly-like mass which could be easily transferred with a pipette. When the plates

were poured, the agar containing the Nile-blue sulphate was melted and the fat emulsion added to the hot agar in the proportion of 1 ml. of the emulsion to 20 ml. of the agar. The agar was allowed to remain hot for a few minutes before it was poured since this intensified the red color of the fat globules. Large colonies of the organisms being tested were secured by touching the surface of the solidified agar with a small loop that had been put into a milk culture of the organisms. The plates were incubated at the appropriate temperature and observations were made at various times, using a hand lens or a wide-field binocular.

Rationale of this test:

As a pH indicator in aqueous solution Nile-blue sulphate is blue at pH 10.1 and red at pH 11.7. Actually Nile-blue sulphate is composed of two dyes, the oxazone fraction being soluble in water, fats and fatty acids, and which is red when oxidized and blue when reduced, and the blue oxazine fraction which is soluble in water and fatty acids but not in fat, and which when reduced forms a colorless or leuco base.

Ordinarily fat is stained red by Nile-blue sulphate. When it is stained blue, using Nile-blue sulphate, the blue color may indicate, either, the reduction of the red oxazone by bacterial products soluble in fat, or, the absorption of the blue oxazine by bacterial products soluble in fat.

We include this observation by Castell and Bryant(18)

in order to point out that the presence of blue-colored fat globules is not necessarily a result of fat hydrolysis as earlier workers believed. Fatty acids produced as a result of fat hydrolysis result in the absorption of the blue oxazine and the fat globule although appearing intact is surrounded by the blue-stained fatty acid.

Either the oxazone or the oxazine may be permanently oxidized to the colorless states by oxidase-producing bacteria or by strong oxidants such as hydrogen peroxide. In our work using the Nile-blue sulphate procedure we did not observe blue-stained globules but in every instance, when the wide-field binocular was used colorless circular areas could be detected within the area of fat alteration. Two possible explanations of these colorless circular areas present themselves; the fat globule may be intact and the Nile-blue sulphate oxidized to the colorless state, or the colorless circular area observed may be merely an artifact, i.e., the impression left in the solidified agar by the fat globule, which has since disintegrated. We have no means available to differentiate these possibilities.

A positive Nile-blue sulphate test probably usually means that some bacterial attack on the fat is under way. To date workers have accepted the absence of red-stained fat globules as evidence of fat alteration probably lipolysis. For the purposes of our preliminary investigation of the various strains of Staphylococcus we have accepted a similar interpretation of the results obtained with this test.

The method employed by us in testing Staphylococcus strains for lipolytic activity was essentially that of Collins and Hammer. The fat emulsion used was made by adding 10 ml. of tributyrin to 90 ml. of 0.5% agar solution. This emulsion was sterilized by autoclave at 15 pounds pressure for 20-30 minutes. To every 100 ml. of melted sterile nutrient agar 5 ml. of this emulsion was added prior to plate pouring. Turner (16) has pointed out that certain strains of Staphylococcus aureus are inhibited by the presence of Nile-blue sulphate 1:8,000. Our initial experiments with this procedure demonstrated that approximately 50 per cent of the Staphylococcus cultures being tested were inhibited by a concentration of Nile-blue sulphate 1:10,000 in the finished medium. To avoid this inhibiting action we prepared our test medium so that the final concentration of Nile-blue sulphate in this medium was 1:100,000. All the strains of Staphylococcus tested by us grew slowly on this medium but growth was not entirely inhibited. Medium was poured in large Petri dishes 14 centimetres in diameter, the object being to test as many strains of Staphylococcus as possible under identical conditions and on the same medium. In this manner it was possible to test at least eight different strains in duplicate on the same plate. A standard loop was used for inoculation. The inoculum was from a healthy surface growth of the particular Staphylococcus strain on plain agar. Giant colonies were prepared by touching a loopful of the inoculum to the surface of the test medium. Plates were incubated at 37°C. for two or three days, then sealed with Scotch tape to pre-

vent drying of the medium. Incubation was continued for thirteen days at 37°C. Daily measurement of the diameter of the area of fat alteration surrounding each giant colony was recorded in centimetres. The area of fat alteration was considered to be that area within which no red-stained fat globules were observed. The measurements recorded after forty-eight hours' incubation and after thirteen days' incubation are given in Chart Ten.

CHART TEN

Lipolytic activity of Staphylococcus

CHART TEN

Incubation Temperature 37°C.

Staphylococcus Strain No.	Incubation Period	
	Forty-eight hours	Thirteen days
3249	0.5 centimetres	1.4 centimetres
3079	0.5	1.1
3297	1.1	2.4
3290	0.8	1.7
1248	0.5	1.1
3278	1.1	1.9
3292	0.8	1.6
1249 No Test		
3288	0.9	1.7
1244	0.6	1.4
3222	0.6	1.3
3230	0.8	2.2
3332	0.8	1.3
34 No Test		
35	1.2	2.2
3334 No Test		
213	1.2	2.0
187	0.3	2.1
3312	1.0	2.2
1251	0.4	2.0
1252	0.4	1.5
3329	0.8	2.3
214	0.8	2.1
55	1.0	3.0
54 No Test		

Summary

We were unable to recognize any relation between the source of Staphylococcus and inhibitory potency. The two most powerfully inhibitory strains were from a wound infection of the buttocks and from food.

Most of the strains of Staphylococcus studied were of golden pigmentation, haemolytic on sheep's blood agar, fermented lactose and mannite and gave a positive tube test for coagulase using human plasma. However, there are several strains which gave strong positive tests in all these respects and which are less efficient inhibitors than strain 35, which was white, only slightly haemolytic, fermented lactose, did not ferment mannite in twenty-four hours but did so in forty-eight hours and was coagulase negative. These observations confirm those in the literature. We have not, however, tested any number of white, non-haemolytic coagulase negative strains. We have not noticed any such difference in colonial form as described by Duliscouet (5).

If we consider the alteration of fat globules, as demonstrated by the Nile-blue sulphate technique, as evidence of lipolysis, then we can say that all the strains of Staphylococcus tested possessed this characteristic. This confirms the observation of Trussell and Weed (19), who tested thirty-eight strains of Staphylococcus and concluded that they exhibit considerable lipolytic activity. The test itself is purely qualitative and until a more accurate technique is available quantitative estimation of lipolytic

activity based on the results obtained by this method must indeed be only relative. It is difficult to establish any relation between the inhibitory potency, of a particular strain of Staphylococcus towards the growth of Mycobacterium, and the lipolytic activity of that strain of Staphylococcus. For example, Staphylococcus strain 55 appeared to possess the greatest lipolytic activity according to our observations. However, this strain was very weakly inhibitory towards the growth of Mycobacterium. On the other hand, Staphylococcus strain 3297 is second to strain 55 in lipolytic activity and is one of the most potent inhibiting strains.

PART THREE

EXTRACTION AND TESTING OF INHIBITORY AGENT FROM STAPHYLOCOCCUS

Introduction

The literature contains numerous references to the possible growth products of Staphylococcus aureus in particular and the genus in general. As we have previously pointed out the production of lipase by Staphylococcus has been studied by many workers. Many other products of the growth of Staphylococcus have been demonstrated. The pigment of Staphylococcus aureus was reported as lipochrome by Schroeter (20) in 1872 and as lipoxanthine in 1889 by Zopf (21). In 1908 Much (22) reported Staphylococcus aureus produced an enzyme which he regarded as thrombokinase. Seo (23) in the same year reported that hippuricase was produced by this species. Mavrojannis (24) reported in 1903 that Staphylococcus aureus and Staphylococcus albus produced a gelatinase, which does not change gelatine sufficiently but that formaldehyde will harden it again, forming "gelatose". Burnet (25) reported in 1925 that Staphylococcus aureus produced catalase. Gosio (26) in 1904 suggested the employment of tellurite as a useful test for differentiating living from dead cells and found that Staphylococcus aureus reduced tellurite markedly. Gloger (27) in 1906 concluded that organisms capable of reducing potassium tellurite produced hydrogen sulphide. Meyer (28) in 1913 reported the production of acid and gas from

from glucosamine by Staphylococcus aureus. Seo (23) reported that Staphylococcus aureus and Staphylococcus albus were able to dissimilate hippuric acid and that benzoic acid resulted. In addition to the above growth products Emmerling (29) reported in 1896 that decomposition of egg albumin by Staphylococcus aureus resulted in the production of formic, acetic and butyric acid, carbon dioxide, skatol, indol, ammonia, phenol, trimethylamine and betain. In the same references Emmerling states that higher fatty acids are produced by Staphylococcus aureus growing on egg albumin medium. The report of the production of higher fatty acids assumes especial significance when we consider the statement by Dubos (30) that even very small amounts of free fatty acid exert a very strong toxic action on Mycobacterium. Kraaij and Wolff (31) reported in 1923 that Staphylococcus aureus splits fat and not lecithin. Sobin and Stahly (32) reported the isolation of delta-carotene, rubixanthin and an ester of rubixanthin from the pigment of Staphylococcus aureus. The complexity of the investigation of growth products of Staphylococcus is clearly illustrated by the references cited above.

The literature regarding the effect of extracts of Staphylococcus aureus on other organisms contains a recent report by Nutini, Kelly and McDowell (33). In 1946 these workers reported that protein free alcoholic extracts of the cells of a single strain of Staphylococcus aureus had an inhibitory action towards Escherichia coli, Aerobacter aerogenes, Staphylococcus aureus and Shigella dysenteriae. In high concentration this extract stimulated the growth of

Staphylococcus aureus and Sh. dysenteriae. Extracts of broth culture stimulated the growth of E. coli when present in concentration of 5 per cent, the other organisms tested were either inhibited or no effect was demonstrable. Extracts of filtrates had essentially a stimulant action towards the growth of all organisms tested.

The report of Duliscouet (5) previously cited deals with the characteristics of an extract of Staphylococcus and the effect of such an extract on the growth of the diphtheria bacillus.

We were unable to find any reference in the literature regarding the effect of Staphylococcus extract on the growth of Mycobacterium.

Experimental

Preliminary experiments suggested that filtrates of broth cultures of Staphylococcus would not yield enough inhibitory material for plate demonstration. These experiments involved growing Staphylococcus strain 187 in an extract broth with Bactopeptone and salt, and in the same with 1 per cent dextrose, for nine days and one day respectively at 37°C. These cultures were then passed through Mandler diatomaceous earth filters. Sterile filter paper patches 1.2 centimetres in diameter were dipped in the filtrates and placed at the centre of heavily inoculated Loewenstein plates. Incubation revealed no inhibition of even the slower growing mammalian strains of Mycobacterium, although when the living organisms

from the same broth culture of Staphylococcus were used inhibition was marked.

Extraction of Staphylococcus bodies was first attempted using strain 187 (at this time we did not know that this strain possessed relatively little inhibitory power). This strain of Staphylococcus was grown on twelve plain agar plates at 37°C. for 36 hours. The confluent growth was harvested by scraping the surface of the plates, about 3.0 ml. of the material was thus obtained. Extraction was carried out according to the procedure employed by Harper (34) for the extraction of penicillinase. The organisms were extracted for one hour at room temperature (20-25°C.) with about 20 ml. of acetone. After filtering through ordinary filter paper, the bodies were again extracted with 15 ml. of acetone for one hour. Filtration was again carried out and extraction with two 10 ml. volumes of ether followed. The ether and acetone extracts were then evaporated to nearly dryness. Filter paper patches were used as previously described, moistened in the acetone extract, the ether extract and the residue consisting of the extracted Staphylococcus bodies. Plates of Loewenstein medium were used for testing these fractions in the manner described above. Neither the acetone extract nor the extracted bodies gave any inhibition. The results for the ether extract are given in Chart Eleven.

CHART ELEVEN

Preliminary results using an ether extract of

Staphylococcus 187

CHART ELEVEN

Acid-fast strain	Maximum inhibition observed during period of growth	Final inhibition observed when maximum growth had occurred
192 M. tuberculosis (var. hominis)	0.4 centimetres	0.2 centimetres
222 M. avium	0.2 centimetres	nil
225 M. smegmatis	0.3 centimetres	nil

Surface Plate Tests of Ether Extracts of Staphylococcus 187

Figures given indicate the distance in centimetres from the edge of the filter paper patch to the edge of the Mycobacterium growth.

Figure Ten shows the inhibition of the growth of Mycobacterium strain 192 (var. hominis) resulting from a filter paper patch soaked with the ether extract of Staphylococcus strain 187.

Possible Effect of pH:

To rule out alteration in pH as a possible factor in the inhibition of the growth of Mycobacterium by Staphylococcus extracts, we prepared a buffer series of solutions having pH from 6.0 to 8.7. Filter paper patches as previously employed were moistened with these solutions. Loewenstein medium was prepared and inoculated as before. No inhibition of Mycobacterium strain 192 (var. hominis) or of M. smegmatis strain 225 was observed. Figure Eleven shows one of this series of plates with the typical uninhibited growth of Mycobacterium.

FIGURE TEN

Filter paper patch soaked with ether extract

of Staphylococcus, golden, haemolytic -- # 187

M. tuberculosis (var. hominis) -- Colonel Belcher Hospital

Inoculated: June 27, 1946

Photographed: September 5, 1946

Medium: Loewenstein

FIGURE ELEVEN

Filter paper patch soaked with buffer solution pH 7.1

M. tuberculosis (var. hominis) -- Colonel Belcher Hospital

Inoculated: July 30, 1946

Photographed: September 5, 1946

Medium: Loewenstein

FIGURE 10

FIGURE 11

Having shown that it was possible to extract from the harvested bodies of Staphylococcus a material inhibitory to the growth of Mycobacterium on Loewenstein medium, it became necessary to attempt to develop as simple and rapid a method for titration of the inhibitory potency of extracts as possible. There are a number of methods in the literature which might be applied to the titration of agents inhibiting the growth of Mycobacterium but the process is uniformly a tedious and long one, and subject to many unexpected difficulties. One of the most promising of the more rapid methods appeared to be Muller's (35) modification of the slide culture method of Pryce (36). In this method a thick suspension of bacilli is prepared by scraping the growth from the surface of a three or four week old culture of Mycobacterium on Loewenstein medium. The harvested growth is suspended by grinding with the gradual addition of sterile distilled water. The suspension thus prepared is centrifuged and the supernatant is mixed with an equal volume of fresh blood serum and immediately spread on glass slides (in the Pryce method) or glass chips (in the Muller modification). The slides are dried in the incubator and then treated with 25 per cent sulphuric acid to kill contaminating organisms. Slides are then placed in culture vials to be incubated. Incubation is carried out at 37°C. The medium contained in the screw capped vials may be:

(a) 5 ml. of sterile distilled water plus 2.5 ml. of citrated human blood

or

(b) 5 ml. of citrated human blood plus 5.0 ml. of 1 per cent saponin solution.

Growth on the slides is stated to be observable after one week's incubation; at this time slides are removed from vials, stained and examined. Slides may be removed, one each day, so that a day by day comparison of the growth may be observed.

The slides which we employed were made by cutting ordinary microscope slides in three lengthwise so that glass strips approximately 5/16 of an inch wide by 3 inches in length were obtained. The suspension of organisms was placed on the terminal quarter of the glass strip, fixed by the drying process and placed in vials. The treatment with sulphuric acid was omitted since we were dealing with a pure culture of Mycobacterium. Slides were placed in the culture vials containing the medium in such a manner that the medium covered the inoculated portion of the slide. Incubation and subsequent observation of slides was as outlined above. Both the Pryce and Muller techniques were attempted several times using both human and sheep's blood in the medium. Initially our intention was to dilute the inhibitory agent, extracted from Staphylococcus, in this medium and thus roughly estimate the inhibitory potency of such extracts. The procedure was abandoned due to two main difficulties. Using the glass strip technique of Pryce we found that following incubation the acid-fast organisms could not be found at the initial site of inoculation but were found at the extreme opposite end of the glass strip. This was probably due to inadequate fixing of the original inoculum and the organisms loosening from the slide during

incubation being carried to the surface of the medium floated upward in the thin film of medium adhering to the glass strip, due to surface tension. Using the glass chip method, the chips entirely immersed in medium, this difficulty did not arise. The second and the greatest difficulty encountered was the inability to obtain a suspension of isolated bacilli for the original inoculum. Tubercle bacilli are notoriously tenacious in clumping. Despite tedious grinding we were unable to break up these clumps. The presence of clumps in the stained specimen following incubation prevented detection of any growth which may have occurred. Chart Twelve is a diagram of the Pryce and Muller technique as employed by us and illustrates the difficulty present in the Pryce method.

CHART TWELVE

Chart Twelve

Slide Culture Technique
Pryce

Chip Culture Technique
Muller

X = Site of Original Inoculum

Y = Site of Organisms after Incubation

Use of Dubos' Medium for Titration of Staphylococcus Extract

A new liquid synthetic medium for the rapid and submerged growth of Mycobacterium as developed by Dubos (37) was next employed. Our first work was performed using a medium of the following composition:

Na_2HPO_4 (anhydrous)	2.5 grammes
KH_2PO_4	1.0 gramme
Sodium citrate	1.5 grammes
Asparagine	1.0 gramme
$MgSO_4.7H_2O$	0.6 gramme
Distilled water	1000 ml.
Tween 80	0.5 ml.

This was dispensed in 1.0 ml. volumes in test tubes, and autoclaved. At first the test tubes were plugged with cotton, later screw capped tubes were used. Finally because of difficulties involved in drying out of the medium even in screw capped vials, and difficulties in studying the character of the growth in such small volumes, we changed to 5.0 ml. amounts and abandoned screw capped tubes in favor of cotton plugged tubes. We also felt that it was better to give freer access of air than screw capped vials allowed.

At first, attention was confined to Mycobacterium tuberculosis var hominis, strain 192, and Staphylococcus aureus, strain 187. This strain of tubercle bacilli reached maximum growth (by macroscopic estimation) in the above medium in 4 to 5 days when we first started using it. We proceeded to make

extracts of Staphylococcus and titrate them as follows:

Extraction Methods:

Method One

Twenty-five to thirty plain agar plates were inoculated confluently on the surface with Staphylococcus and incubated at 37°C. for 24 to 96 hours. The growth was harvested using a sterile metal scraper, little or no agar being taken. The harvested organisms were placed in a sterile mortar and di-ethyl ether added. Grinding was carried out by hand, using a sterile pestle, until the staphylococcal bodies assumed a dry granular character (about two hours being required). The ether extract was then filtered through fine grade filter paper into a distilling flask. At this point the extract consisted of 75 to 100 ml. of a pale yellow liquid having an odour suggestive of buckwheat honey.

This extract was evaporated under reduced pressure, using a water bath at 45 to 50°C., until the volume approximated 10 ml. Ten millilitres of sterile distilled water was then added, forming a yellowish opaque emulsion, sometimes with macroscopic oily droplets. Concentration was continued with shaking until the volume was again about 10 ml.

In one case a similar extraction using acetone was carried out on the residue remaining after the ether extraction. This acetone extract was concentrated in a similar manner.

Method Two

Seven hundred fifty millilitres of brain heart infu-

sion broth (Difco) inoculated with Staphylococcus and incubated at 37°C. for 24 hours and at room temperature (20-25°C.) for an additional 48 hours was serially extracted with five 150 ml. volumes of di-ethyl ether. The extract was separated by centrifugation and concentrated in the manner described under Method One.

Method Three

Staphylococcus growth was harvested as in Method One. The harvest was placed in the extraction thimble of a Soxhlet Continuous Extraction Apparatus and extracted with 75 ml. of di-ethyl ether at 45 to 50°C. continuously for four hours. This extract was concentrated in the manner described in Method One.

Titration Methods:

One cubic centimetre volumes of the aforementioned Dubos' medium were used in screw capped test tubes.

Doubling dilutions of extracts were made with sterile precautions in these amounts. Each tube was then inoculated with one drop of a 1:10,000 dilution of the tubercle bacilli grown in Dubos' medium for several days. The growth in Dubos' medium had a characteristic almost ropy character, but was easily evenly suspended by shaking. Incubation was carried out at 37°C. and readings made daily. Slowing of growth as well as complete inhibition was often observed.

Results:

Chart Thirteen presents a summary of the details of some of seventy odd titrations done at various ages of the extracts (which were stored at 6-10°C.) under various conditions.

CHART THIRTEEN

Titration of *Staphylococcus* extracts

against *Mycobacterium*

CHART THIRTEEN

Some of the Inhibitory Titres of Various Extracts

Identifying Number of Extracts	Method of Extraction *****	Special Features of Extract	Titre: * In upper right hand corner is age of extract in days	Titre: * In upper right hand corner is age of extract in days	Titre: * In upper right hand corner is age of extract in days
a	1		**** 128 0	128 1	200,000 8
a	1	Filtered through asbestos pad		128 1	12,000 8
b	2		128 0	128 1	25,000 8
b	2	Filtered through asbestos pad		128 1	25,000 8
c	1	Acetone extract of ether-extracted bodies	128 0	0 1	
c	1	Filtered through asbestos pad	16 0	32 - 64 1	
EESox 1	3			64 18	
EESox 2	3			128 14	1024 21
EESox 3	3		1024 0	64 9	
EESox 3	3	Extract held at 100° C. for 15 minutes		32 9	
EESox 4	3		4096 0	128 5	1024 15
EESox 4	3	Extract held at 100° C. for 15 minutes		16 5	
EESox 4	3	Each tube contained 0.5% glycerine		8192 22	
EESox 5	3		256 0	1024 1	256 2
EEG 6	1		4096 0	33,000 2	
EEG 6	1	**		0 6	
EEG 6	1	10% normal rabbit serum in each tube		8 6	
EEG 6	1	50% normal rabbit serum in each tube		irregular 6	
EESox 7	3		1024 0	256 1	
EEG 8	1			512 5	
EEG 8	1	10% sheep serum in each tube		4 7	
EEG 8	1	10% guinea pig serum in each tube		0 11	
EEG 8	1	10% human serum in each tube		0 11	
EESox 9	3			256 5	
EEG 10	1		512 0		
Ether		***	16 0		

* The greatest dilution of extract present which completely inhibited growth. Titrations were usually held about fourteen days.

** Added 0.1 c.c. of 1-10 extract to 0.9 c.c. normal rabbit serum. After allowing to stand 10 minutes, made dilutions in Dubos titration series.

*** 75 c.c. ether reduced to 10 c.c. volume by evaporation, 10 c.c. distilled water added, reduced again to 10 c.c. volume by evaporation.

**** No higher dilutions were tried.

***** See explanation in text.

Summary

Chart Thirteen illustrates certain properties of the extracts of Staphylococcus. These are:

(a) The titres of the extracts vary markedly.

(b) There are several instances of inconsistency in two titrations of the same extract at different times, (e.g., EESox 2 and EEG 6).

(c) The introduction of blood serum into the medium practically wipes out the inhibitory effect of the extracts.

(d) Filtration through an asbestos filter pad removes most of the inhibitory effect of the extracts.

(e) Heating to 100°C. for 15 minutes removes some of the inhibitory effect of the extracts.

It is clear, however, that ether extraction of the bodies of Staphylococcus strain 187 yields a preparation which under certain conditions is markedly inhibitory to the growth of Mycobacterium strain 192.

Our titration results obviously emphasized the necessity of further standardization of the titration procedure, and the method of extraction. We therefore set up an experiment in which it was proposed to make a number of extractions and titrations under carefully standardized conditions. In order to avoid changes of the tubercle bacilli due to continued passage through Dubos' medium, we returned to a Loewenstein culture of Mycobacterium strain 192. To our surprise, none of the

fifty-six titrations on seven new extracts showed any growth at all, even in control tubes containing no inhibiting agent. We then re-investigated the Dubos-passaged strain (which by this time had been passed through Dubos' medium nine times) and found that the strain would now grow up overnight on Loewenstein medium and even on plain agar medium, and that it had lost its pathogenicity for guinea pigs. We have tried to reproduce this apparent change in character of Mycobacterium strain 192 without success. We are unable to say whether it really was a change, or whether at some stage we mixed it with a fast-growing, non-pathogenic strain.

Because of these difficulties we turned to experiments designed to reveal the reasons for the absence of growth in our latest titrations. Studies of Dubos' recently published papers on the subject helped us considerably. The main difficulty which is emphasized by Dubos is the hydrolysis of Tween 80, with the production of small amounts of long chain unsaturated fatty acids. These fatty acids inhibit the growth of tubercle bacilli (30, 38). This inhibition may be countered by the introduction of a small amount of serum albumin into the medium. Tween 80, the commercial product always contains small amounts of fatty acids, and when the medium is made up, hydrolysis tends to occur slowly so that if albumin is not used the medium may become inhibitory over a period of time.

Our work confirmed the observation of Dubos' et al. that smaller inocula would suffice in the presence of albumin. In its absence, using the slow-growing strain of Mycobacterium

192, a drop of undiluted culture as inoculum for each 5.0 c.c. volume of medium was required before growth could be consistently obtained; whereas with albumin, a drop of culture diluted as much as 1 in 100 sufficed. However, we are of the opinion (but on the basis of slight experimental evidence) that if Tween 80 is purified by a method reported by Davis, to be published shortly (39), and, used without albumin, growth is better, than with unpurified Tween 80 though not usually as rapid as when albumin is used.

Our study of the factors influencing the growth of Mycobacterium strain 192 in Dubos' medium led us to the following conclusion. Our main error was in using too small an inoculum. (It will be remembered that a drop of a 1:10,000 dilution of a culture in Dubos' medium was used.) In such titrations it is our opinion that the Tween 80 that is used should be freed of uncombined fatty acid by purification. It is necessary to use a medium without albumin in the titration of Staphylococcus extracts because the albumin removes the inhibitory quality of the extracts. Five cubic centimetre volumes of medium per titration tube are better than smaller volumes.

The medium used in the later titrations had the following formula (one of Dubos' modifications of his first medium) (40):

$Na_2HPO_4.12H_2O$	6.3 grammes
KH_2PO_4	1.0 gramme
Asparagine	2.0 grammes
Casitone (Difco)	2.0 grammes

Ferric ammonium citrate	1 ml. of a 5% solution
$MgSO_4.7H_2O$	1 ml. of a 1% solution
$CaCl_2$	1 ml. of a 0.05% solution
Zinc sulphate	1 ml. of a 0.01% solution
Copper sulphate	1 ml. of a 0.01% solution
Distilled water	to 1,000 ml.
Tween 80	5 ml. of a 10% solution

Ethanol extracts of Staphylococcus were attempted. The method of extraction used was that of Jansen and Hirschmann (41). Unfortunately, it is difficult not to leave some living staphylococci using this method of extraction unless considerable heat is employed. As we have demonstrated, heating affects the inhibitory potency of the extracts. We feel that we were able to obtain slight inhibition using the ethanol extraction method despite heating.

A point which sometimes became quite bewildering was the variation in the macroscopic character of the growth of Mycobacterium strain 192 in the titration series. Originally, as observed in earlier titrations, there appeared a small mound of sediment in the bottom of the tube which upon gentle shaking swirled upward and became evenly distributed. It seemed, however, to have a certain degree of tenacity and the word "ropy" described it fairly well. In titration series, however, the strain sometimes appeared to grow in flakes which settled to the bottom of the tubes and although readily suspended by shaking, the individual flakes were difficult to break up. Often some of the tubes in a titration series would yield one type of growth while others looked entirely dissimilar. Had

there been any consistency concerning the presence, absence or amount of the inhibitory agent when a particular type of growth appeared, it would have been less confusing.

We felt it would be interesting to see to what extent oleic acid would inhibit the growth of Mycobacterium strain 192 under the conditions in our experiments since Dubos emphasizes the inhibiting action of unsaturated fatty acids. It seems likely that the inhibition of Mycobacterium growth by Staphylococcus on Loewenstein medium might be due to such acids (29). In Loewenstein plate experiments using filter paper discs soaked in 100 per cent oleic acid an initial inhibitory distance (measured from the edge of the filter paper disc to the nearest acid-fast growth) of 0.25 centimetres and a final inhibitory distance of 0.15 centimetres was obtained. (Compare with results shown in Chart Eleven.) Ten per cent oleic acid suspended in Dubos' medium and tested by the filter paper disc method gave an initial and final inhibitory distance of 0.05 centimetres. A filter paper disc soaked in Dubos' medium gives no inhibition, nor does 1 per cent or smaller amounts of oleic acid. We consider that the total inhibitory action of the Staphylococcus extracts can hardly be due to fatty acid alone, since inhibitory potency was considerably lessened by heating, pointing to possible enzyme activity.

With regard to the diffusibility of the inhibitory agent, tubercle bacilli were inoculated into Dubos' medium in small Erlenmeyer flasks. In each flask was also placed a loop of cellophane tubing containing plain broth inoculated with Staphylococcus. Dubos' medium, with and without albumin, were

both used. These flasks were incubated at 37°C. The tubercle bacilli grew well in the Dubos' medium whether the cellophane tubing contained Staphylococcus or not. Thus, there was no significant amount of inhibitory agent excreted into the culture medium by Staphylococcus which would pass through the cellophane barrier. This technique of testing for diffusibility has been described by Heatley and Florey (42) in connection with colicine.

SUMMARY

Summary

1: Eleven strains of acid-fast bacilli were tested, on Loewenstein medium, for susceptibility to inhibition by staphylococci. It was observed that there are marked differences among strains of acid-fast bacilli in susceptibility to inhibition by staphylococci.

2: In general, the order of susceptibility is: human, bovine, other strains.

3: In general, but with exceptions, slow-growing strains of Mycobacterium are inhibited to a greater extent than fast-growing strains.

4: Twenty-seven strains of Staphylococcus were tested for inhibitory potency toward the growth of Mycobacterium. There is a marked variation among Staphylococcus strains in inhibitory potency.

5: Inhibitory potency of Staphylococcus strains could not be related to the source, microscopic or colonial morphology, staining reaction, haemolytic activity, lactose or mannite fermentation, coagulase production or lipolytic activity of the Staphylococcus strains.

6: There is a suggestion that there exists a high degree of specificity in the relationship between Staphylococcus and Mycobacterium strains.

7: It is possible, using di-ethyl ether, to extract an inhibi-

tory material from Staphylococcus bodies harvested from plain agar medium. Extracts from whole broth cultures of Staphylococcus are also potent.

8: The inhibitory material is removed by filtration through an asbestos filter pad.

9: The inhibitory action of such extracts is abolished largely, if not completely, in the presence of serum albumin. For this reason, titration in Dubos' medium must be performed in the absence of albumin. (The presence of coagulated egg white in Loewenstein medium does not abolish the inhibitory activity.)

10: Heating of extracts at 100°C. for 15 minutes materially reduces the inhibitory potency.

11: When Mycobacterium and Staphylococcus strains are grown in liquid media separated only by a layer of cellophane, no inhibition of Mycobacterium is observed.

REFERENCES

REFERENCES

(1) JENNINGS, M.A. and SHARP, A. E., Antibacterial Activity of the Staphylococcus, Nature 159: 133-134, (1947)

(2) DUJARDIN-BAUMETZ, E., Action antibiotique d'une varie te de staphylocoque a l'egard des bacilles gram-Positifs et acido-resistants, Compt. rend. Soc. de biol. 110: 1210-1213, (1932)

(3) McPHEDRAN, F. M., LANE, E. L. and CROSSON, J. W., Further Observations on Interrelations of Tuberculous and Non-tuberculous Pulmonary Infections, with Particular Reference to Staphylococcus Reactivity, Tr. Am. Clin. and Climetol. A. (1938) 54: 19-41, (1939)

(4) ARENA, A. R., Accion inhibidora de un estafilococo sobre el cultivo del bacilo de Koch (nota previa), Rev. Asoc. med. argent. 58: 1026-1027, (1944)

(5) DULISCOUET, R., Un antagoniste microbien nouveau; la staphyline antidiptherique, Presse med. 53: 654-655, (1945)

(6) WAKSMAN, S. A., Microbial Antagonisms and Antibiotic Substances, 60-64, (1945), The Commonwealth Fund, New York

(7) GARRÉ, C., Über Antagonisten unter den Bakterien, Centrabl. f. Bakt. 2: 312-313, (1887)

(8) FLEMING, A., On the Antibacterial Action of Cultures of

a Penicillium, with Special Reference to Their Use in the Isolation of B. influenzae, Brit. J. Exper. Path. 10: 226-236, (1929)

(9) ABRAHAM, E. P., BAKER, W., CHAIN, E., FLOREY, H. W., HOLIDAY, E. R. and ROBINSON, R., Nitrogenous Character of Penicillin, Nature 149: 356, (1942)

(10) ABRAHAM, E. P., CHAIN, E. and HOLIDAY, E. R., Purification and some Physical and Chemical Properties of Penicillin; with a Note on the Spectrographic Examination of Penicillin Preparations, Brit. J. Exper. Path. 23: 103-120, (1942)

(11) CHAIN, E., FLOREY, H. W., GARDNER, A. D., HEATLEY, N. G., JENNINGS, M. A., ORR-EWING, J. and SANDERS, A. G., Penicillin as a Chemo-therapeutic Agent, The Lancet 239: 226-228 (1940)

(12) STOKES, J. L. and WOODWARD, C. R. Jr., The Isolation of Soil Bacteria that Produce Bactericidal Substances, J. Bact. 43: 253-263, (1942)

(13) SCHOENTAL, R., The Nature of the Antibacterial Agents Present in Pseudomonas pyocyanea Cultures, Brit. J. Exper. Path. 22: 137-147, (1941)

(14) CHAPMAN, G. H., BERENS, C., NILSON, Edith L. and CURCIO, Lillian G., The Differentiation of Pathogenic Staphylococci from Non-pathogenic Types, J. Bact. 35: 311-334, (1938)

(15) EIJKMAN, C., Ueber Enzyme bei Bakterien und Schimmelpilzen, Centrabl. f. Bakt., Abt. 1, 29: 841-848, (1901)

(16) TURNER, R. H., The Action of Bacteria on Fat, J. Infect. Diseases 44: 126-133, (1929)

(17) COLLINS, M. A. and HAMMER, B. W., Types of Lipolysis brought about by Bacteria as Shown by Nile-blue Sulphate, J. Bact. 27: 487-496, (1934)

(18) CASTELL, C. H. and BRYANT, L. R., Action of Micro-organisms on Fats, Iowa State College, J. Sc. 13: 313-328, (1939)

(19) TRUSSELL, R. E. and WEED, L. A., The Lipolytic Action of Staphylococci on some pure Triglycerides, J. Bact. 33: 381-388, (1937)

(20) SCHROETER, J., Ueber einige durch Bacterien gebildete Pigmente. Cohn's Beiträge zur Biologie der Pflanzen, 1 (Heft 2): 109-126, (1872)

(21) ZOPF, W., Ueber das mikrochemische Verhalten von Fettfarbstoffen und fettfarbstoffhaltigen Organen, Ztschr. f. wissensch. Mikr. 6: 172-184, (1889)

(22) MUCH, H., Über eine Vorstufe des Fibrinfermentes in Kulturen von Staphylokokkus aureus, Biochem. Ztschr. 14: 143-155, (1908)

(23) SEO, Y., Über die Hippursäurespaltung durch Bakterien und ihre Bedeutung für den Nachweis von Benzoesäure und Glykokoll in Harne, Arch. Expt. Path. u. Pharmakol. 58: 440-449, (1908)

(24) MAVROJANNIS, Sur la nature des diastases microbiennes liquefiant la gelatine, Compt. Rend. Soc. Biol. (Paris) 55: 1605-1606, (1903)

(25) BURNET, F. M., Hydrogen Peroxide and Bacterial Growth. Aust. J. Expt. Biol. and Med. Sci. 2: 66-76, (1925)

(26) GOSIO, B., Sulla decomposizione di sali del telluric per opera dei micro-organismi, Atti R. Accad. Lincei, Rend. Cl. Sci. Fis., Mat. e Nat. 13: 422-427. After Bul. Inst. Pasteur 2: 523-524, (1904)

(27) GLOGER, R., Kalium tellurosum in der Medizin und Hygiene, Centrabl. Bakt. Abt. 1, 40: 584-590, (1906)

(28) MEYER, K., Über das Verhalten einiger Bakterienarten gegenüber d-Glucosamin, Biochem. Ztschr. 5: 297-299, (1913)

(29) EMMERLING, O., Beitrag zur Kenntnis der Eiweissfäulniss, Ber. Deut. Chem. Gesell. 29: 2721-2726, (1896)

(30) DUBOS, R. J., Effect of Long Chain Fatty Acids on Bacterial Growth, Proc. Soc. Exper. Biol. and Med. 63: 56-58, (1946)

(31) KRAAIJ, G. M. and WOLFF, L. K., Over de splitsing van lipoïden door bacteriën, K. Akad. Wetensch. Amsterdam 32: 624-625, (1923)

(32) SOBIN, B. and STAHLY, G. L., Isolation and Absorption Spectrum Maxima of Bacterial Carotenoid Pigments, J. Bact. 44: 265-276, (1942)

(33) NUTINI, L. G., KELLY, T. A. and McDOWELL, Margaret, Effect of Staphylococcus aureus Extracts on Various Bacteria, J. Bact. 51: 533-538, (1946)

(34) HARPER, G. J., Inhibition of Penicillin in Routine Culture Media, The Lancet 245: 569-570, (1943)

(35) MULLER, H., On the Use of a Modification of Pryce's Slide Culture Method for the Estimation of the Bacteriostatic Power of Chemicals on the Tubercle Bacillus, J. Path. and Bact. 56: 429-433, (1944)

(36) PRYCE, D. M., Sputum Film Cultures of Tubercle Bacilli: a Method for the Early Observation of Growth, J. Path. and Bact. 53: 327-334, (1941)

(37) DUBOS, R. J., Rapid and Submerged Growth of Mycobacteria in Liquid Media, Proc. Soc. Exper. Biol. and Med. 58: 361-362, (1945)

(38) DUBOS, R. J., DAVIS, B. D., MIDDLEBROOK, G. and PIERCE, Cynthia, The Effect of Water Soluble Lipids on the Growth and Biological Properties of Tubercle Bacilli, Am. Rev. Tuberc. 54: 204-212, (1946)

(39) DAVIS, B. D., The Preparation and Stability of Fatty Acid-free Tween 80, Laboratories of the Rockerfeller Institute for Medical Research (Personal Communication, to be Published)

(40) DUBOS, R. J., MIDDLEBROOK, G. and PIERCE, Cynthia, Composi-

tion of Media for Growth of Mycobacteria (Personal Communication)

(41) JANSEN, E. F. and HIRSCHMANN, D. J., Subtilin - Antibacterial Product of Bacillus subtilis: Culturing Conditions and Properties, Arch. Biochem. 4: 297-309, (1944)

(42) HEATLEY, N. G. and FLOREY, H. W., An antibiotic from Bacterium coli, Brit. J. Exper. Path. 27: 378-390, (1946)

APPENDIX

APPENDIX 1

LOEWENSTEIN MEDIUM

Salt Base:

Potassium phosphate (monobasic)---- 1 gramme

Sodium citrate---------- 1 gramme

Asparagin---------- 3 grammes

Magnesium sulphate ($7H_2O$)--------- 1 gramme

Distilled water--add to---------- 1000 ml.

The above mixture is heated gently until dissolved.

Preparation of Loewenstein Medium:

Salt base---------- 300ml.

Potato Starch---------- 12 grammes

Glycerol---------- 25ml.

The above mixture is heated in water bath with occasional shaking, until dissolved.

Add:

Whole eggs (fresh) ---------- 8

Egg yolks---------- 2

Shake until homogenous.

Add:

Malachite Green (2% aqueous solution)--10ml.

This medium is plated using a funnel and tube set up.

Medium is sterilized by inspissation or autoclaving.